Interaction of Rat Ascites Hepatoma Cells with Cultured Mesothelial Cell Layers: A Model for Tumor Invasion

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ABSTRACT

Interactions of rat ascites hepatoma cells with primary cultured layers of rat mesentery-derived cells were studied. The mesentery-derived cells were isolated from rat mesentery and cultured in Eagle's minimum essential medium with a 2-fold concentration of amino acids and vitamins supplemented with 10% calf serum. The primary cultured cells, consisting mainly of mesothelial cells in polygonal shape, forms a "paving stone" sheet. Upon seeding the tumor cells on the mesentery-derived cell layers, three different types of tumor cell growth were observed. Type 1 was the formation of piled-up tumor cell nests on mesothelial cell layers. Type 2 was the formation of flattened tumor cell islands underneath mesothelial cell layers. This island formation was clearly observed under a phase contrast microscope 2 days after the tumor cell seeding. Protrusion of cellular processes of the tumor cells beneath mesothelial cells was occasionally seen. Type 3 was the growth of tumor cells in suspension. These types of tumor cell growth closely resemble those in the peritoneal cavity seen in vivo. These results indicate that the system described in this paper can provide a useful model to study tumor cell invasion.

INTRODUCTION

Malignant tumor cells are capable of invading into surrounding normal tissues and spreading to form secondary tumors (metastases) at distant host sites. Invasion and metastasis seem to proceed by a series of complex sequential steps which has been studied extensively (1-6), but the mechanisms by which they occur are yet unknown.

Several in vitro model systems including isolated bladders have been used to study the mechanisms by which invasion and metastasis occur. However, these model systems are not analogous to the in vivo situation. In these model systems, tumor cell invasion is observed in a 3-dimensional matrix, which is usually not encountered by tumor cells in vivo.

In this paper we describe a culture system for studying an invasion of tumor cells into mesothelial cell layers. This system was developed to study the mechanisms by which tumor cells invade the peritoneum, invade mostly into the mesentery, and appear in the blood stream. Retention of ascites fluid occurs and the host is killed in 2 to 3 wk.

On implantation of AH 130 cells in the peritoneal cavity, the tumor cells must first interact with mesothelial cells surrounding the cavity. The interaction seems to be an initial step of a complex sequence of tumor cell invasion into mesothelial tissues and of subsequent intravasation. We have recently developed an in vitro model system to examine the interaction of tumor cells with mesothelial cells which were isolated from the rat mesentery and cultured as a monolayer. Seeding AH 130 cells on the monolayer, we observed invasive growth of the tumor cells underneath the cultured mesothelial cell monolayers which mimicked the in vivo invasion by the tumor cells into rat peritoneum.

MATERIALS AND METHODS

Isolation and Culture of Mesentery-derived Cells. Peritoneal cavities of rats (Dondry strain) were opened under ether anesthesia. Window-like transparent triangular sheets of mesentery were seen bordered by two adjacent jejunal arteries which were located within streaks of adipose tissue. The center of each sheet was picked up with small forceps and the transparent part of mesentery was cut along the border of the sheet with a scissors. About 15 mesenteric sheets could usually be obtained from a rat. The sheets were then incubated at 37°C for about 20 min in 0.25% trypsin (1:250; Difco) in PBS. After the digestion, the filtrate was centrifuged to pellet mesentery-derived cells. Examination of the digested sheets after staining with hematoxylin-eosin indicated considerable removal of mesothelial cells from the mesenteric sheets.

Isolated sheets were then incubated at 37°C for about 20 min in 0.25% trypsin (1:250; Difco) in PBS. After the digestion, the filtrate was centrifuged to pellet mesothelial cells. Examination of the digested sheets after staining with hematoxylin-eosin indicated considerable removal of mesothelial cells from the mesenteric sheets. Connective tissues and blood vessels remained in the sheets histologically almost intact, indicating selective detachment of mesothelial cells. The mesothelial-derived cells (1 x 10^6) were inoculated in a 35-mm plastic tissue culture dish (Corning Glass Works, Corning, NY) and cultured in EM supplemented with 10% calf serum in an atmosphere of 5% CO_2 in air at 37°C.

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The abbreviations used are: PBS, phosphate-buffered saline (NaCl, 8 g/liter, KCl 0.2 g/liter, Na_2HPO_4 12 H_2O, 2.9 g/liter, and KH_2PO_4 0.2 g/liter) pH 7.4; EM, Eagle's minimum essential medium with a 2-fold concentration of amino acids and vitamins (Biken, Osaka, Japan); M-cell, cultured, mesentery-derived cell; AH 130 cells, rat ascites hepatoma cells, AH 130.
grew forming a monolayer. Primary cultured cells were used throughout the experiments.

Tumor Cells, Conditioned Medium, and Ascites Fluid. AH 130 cells were maintained by successive transplantation into the peritoneal cavity of rats of Donryu strain. The cells were obtained usually 7 days after the transplantation and cultured in EM supplemented with 10% calf serum for 1 wk before use in order to eliminate contaminated macrophages and neutrophils. The cultured tumor cells were confirmed to be transplantable in rat peritoneal cavity. Conditioned medium was prepared from 3 days' culture of AH 130 cells. The medium was dialyzed against EM supplemented with 10% calf serum before use. Ascites fluid was separated from the tumor cells by centrifugation at 10^4 x g for 20 min.

Coculture of AH 130 Cells with the Mesentery-derived Cells. When the mesentery-derived cells grew to confluent state, cultured AH 130 cells (1.2 x 10^6) were seeded on the monolayer and cultured in EM supplemented with 10% calf serum.

Microscopic Observations. Cells were examined by a phase-contrast microscope after they were cultured for various lengths of time. In some tumor cell-seeded samples, AH 130 cells suspended in culture medium were carefully removed and the remaining cell layers were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in the same buffer at room temperature for 1 and 2 h, respectively. The fixed cells were rinsed with water and stained in block with 1% uranyl acetate. The cell layers were then dehydrated in a series of increasing concentrations of ethanol and embedded in Epon 812. Epon-embedded cell layers were sectioned perpendicularly to the M-cell monolayer plane. The sections were stained with 1% methylene blue in 1% sodium borate and observed under a light microscope. Mesentery-derived cells removed from tumor-bearing rats were rinsed thoroughly in 0.85% NaCl, fixed in 10% formalin in PBS and embedded in resin (Technovit 7100; New Metals Chemicals Corp., Tokyo, Japan). The embedded mesenteries were sectioned perpendicularly and stained with hematoxylin and eosin. Mesenteries from normal rats were carefully stretched over glass slides. The whole preparation was dried, fixed in 10% formalin in PBS, and stained with hematoxylin and eosin.

For electron microscopic observations of M-cell monolayer and rat liver, the samples were fixed, dehydrated, embedded, and sectioned by the same procedure as for the tumor cell-seeded samples. The sections were stained with uranyl acetate and lead nitrate and examined under a JEM 100 CX transmission electron microscope.

Acid phosphatase was stained by the method of Janckila et al. (28). Keratin was stained by the peroxidase-anti-peroxidase technique using anti-human keratin rabbit IgG after the cells were treated with 1% Triton X-100 (29). Phagocytosis by the mesentery-derived cells was examined by the uptake of carbon particles after culturing the cells for 2 h in culture medium containing Pelikan ink (1:30 dilution). Assays. The number of cells was counted with either a Coulter Counter or hemocytometer. The number of flattened tumor cell islands was counted under a phase-contrast microscope. A circle (7 mm diameter) was drawn at the center of the outside of the culture dish bottom and the circle was divided into 18 equal lengths of arcs. Since the tumor cell islands were distributed almost evenly on a culture dish, the number of islands in one visual field (1.13 mm^2) adjacent to each arc was counted and the total number in 18 visual fields was expressed as islands per cm^2.

Isolation and Culture of AH 130 Cells from Host Blood. The presence of AH 130 cells in circulating blood of tumor-bearing rats was confirmed morphologically by examining a blood smear after collecting tumor cells (see below). Injection of the host blood into normal rat peritoneal cavity led to the development of the ascites tumor. To isolate circulating AH 130 cells, host blood was obtained by heart puncture and diluted twice with PBS. The diluted sample was layered on an equal volume of Ficoll-metrizoate solution (Muto Pure Chem., Ltd., Tokyo, Japan) and centrifuged at 400 x g for 30 min. Cells recovered from the interface were collected. These consisted of AH 130 cells and mononuclear cells of the host. Preliminary experiments showed that AH 130 cells were recovered from the interface. The collected cells were cultured for about 1 mo to obtain a sufficient number of tumor cells. The tumor cells grew mostly in suspension, whereas the mononuclear cells adhered to culture dishes and did not grow during the culture. Since the cells growing in suspension contained virtually no host cells, they were used as AH 130 cells from host blood.

 Autoradiography. AH 130 cells were incubated at 37°C for 20 h in leucine-free EM supplemented with 10% calf serum to which l-[4,5-^3]H]leucine, 5 μCi/ml (5 Ci/mmol; Amersham) was added. The labeled tumor cells were then chased for 6 h in fresh culture medium, washed twice with the medium, and seeded on mesentery-derived cell layers. After 12 h coculture, AH 130 cells suspended in the culture medium were removed, and the remaining cell layers were fixed on 10% formalin in PBS, covered with NR-M2 liquid emulsion (Konishiroku Photo. Ind. Co. Ltd., Tokyo, Japan), exposed for 1 wk, developed, and stained with hematoxylin solution.

RESULTS

Mesentery-derived Cells. The cultured mesentery-derived cells had an epithelial appearance characteristic of mesothelial cell (Fig. 1, M-cells). Histochemical and functional examinations showed that the cells were positive in acid phosphatase and immunoreactive keratin stainings and negative in Pelikan ink phagocytosis. These characteristic features of the cultured cells excluded the possibility that other cell types like macrophages, fibroblasts, endothelial cells, muscle, and blood cells were contaminated (28–30). M-cells were polygonal in culture, forming a "paving stone" sheet when confluent. The nucleus with prominent nucleoli was round or oval and centrally located. Many mitochondria around the nucleus and well-developed rough endoplasmic reticulum were seen under a transmission electron microscope (Fig. 2 a and b). Bundles of microfilaments were observed in the cytoplasm, especially at the cell periphery parallel to the plasma membrane. Junctional complexes were often seen between two adjacent M-cells (Fig. 2c). In comparison, transmission electron micrographs of a section through the liver with mesothelial cells lining the peritoneal cavity are shown in Fig. 2 d and e. Microvilli and micropinocytic vesicles on the cell surface, a large amount of endoplasmic reticulum, and junctional complexes between the cells were seen. M-cells grew with a doubling time of about 20 h and formed a monolayer in intimate contact with each other.

Coculture of AH 130 Cells on M-cell Layers. AH 130 cells, when cultured in plastic dishes, did not adhere to substrate but
rather grew in suspension. However, when the cells \((1.2 \times 10^5/\text{dish})\) were seeded on the cultured M-cell layers, approximately 50% of the tumor cells adhered to M-cell layers about 20 h after the seeding. Most of the cells attached at or near junctional regions between adjacent M-cells. The attached cells were round and seemed to be not strongly adhered, since vigorous pipeting could easily detach the cells.

On further culturing two types of growth of the adherent tumor cells were seen (Fig. 3) in addition to the third type of tumor cell growth in suspension. The first type of tumor cell growth was seen as piled-up tumor cell nests on M-cell layers \([4.26 \pm 1.18\% \text{ (SD)}\) of the adherent cells]. The second type was characterized by a flattened configuration of tumor cell islands (more than 4 cells/island) which spread parallel to M-cell monolayers \((2.20 \pm 0.32\% \text{ of the adherent cells}; \text{invasive tumor cell growth}; \text{see below})\). Their nuclei and nucleoli were clearly seen under a phase-contrast microscope. This type of growth was easily distinguished from the piled-up growth of tumor cells. The development of flattened tumor cell islands was shown in a series of photographs taken at different times after tumor cell seeding (Fig. 4). Penetration of the tumor cells beneath M-cells was seen in Fig. 4a (see also Fig. 6d). Fig. 4b shows the completion of the penetration, and the subsequent division of the penetrated tumor cells was seen in Fig. 4c and d (see also Fig. 6c). Autoradiographic studies in which the tumor cells were prelabeled with L-[4,5-\(^3\)H]leucine showed that the radioactive grains located mostly in the flattened cells, although there seemed to be a slight reincorporation of the isotope by M-cells (Fig. 5). This result together with the observation shown in Fig. 4 indicated that the flattened cell islands consisted of AH 130 cells. These two types of tumor cell colonies (the piled-up and flattened colonies) usually developed separately, indicating independent origins of tumor cell growth.

When the cocultured cells were fixed \textit{in situ}, embedded in resin, and examined by cutting perpendicularly to M-cell layers, the flattened tumor cell islands were observed underneath M-cell layers (Fig. 6c). The extension of cellular processes of tumor cells under adjacent M-cells was occasionally seen (Fig. 6d). The \textit{in vitro} tumor cell growth on M-cell layer is very similar to the \textit{in vivo} piled-up growth of AH 130 cells (Fig. 6a and b) and the \textit{in vitro} growth underneath M-cells resembles \textit{in vivo} invasion of the tumor cells in host peritoneal cavity (Fig. 6c).
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Fig. 4. Growth of flattened tumor cell islands. Phase-contrast micrographs were taken at 3.5 h (a), 7 h (b), 22 h (c), and 48 h (d) after seeding the tumor cells. Arrows, time-dependent growth of tumor cell islands. X 200.

Since AH 130 cells in host blood were thought to be the ones that had invaded into blood vessels through host mesothelial and submesothelial layers, they could be highly invasive. When the tumor cells recovered from host blood were seeded on cultured M-cell layers, flattened tumor cell islands were formed 15 times more frequently (1544 ± 370/cm²) than when AH 130 cells obtained from the peritoneal cavity were seeded (104 ± 53/cm²). In this special experiment the tumor cells from host blood were cultured for about 1 mo to obtain a sufficient number of tumor cells. Seeding of AH 130 cells from the peritoneal cavity that had been cultured for 1 mo did not result in the increase of the number of tumor cell islands. When cultured on plastic culture dishes in the absence of M-cells, the blood-born tumor cells did not adhere to substrate, indicating that this AH 130 cell population did not differ from original tumor cell population with regard to affinity for plastic.

Morphological Change of M-cells by AH 130 Cells. Two to 3 days after seeding the tumor cells, M-cells showed prominent morphological changes preferentially at the marginal area of layers, a change from polygonal to spindle shape and a partial retraction of the cell edges. These spindle-shaped M-cells joined together and formed circles (Fig. 7a). The morphological change was much less obvious at the central area of layers where M-cells were in close contact with each other.

To examine whether direct contact of the tumor cells with M-cells is essential to cause the retraction, the medium conditioned by AH 130 cell culture was tested. It was found that a similar retraction of M-cells occurred when M-cells were cultured for about 20 h in the conditioned medium. Such morphological change was also observed 4 to 20 h after the addition of 50% tumor ascites fluid (Fig. 7b). Parallel experiments showed that normal rat serum did not cause any retraction of M-cells. These indicate the presence of a factor capable of inducing M-cell retraction in the conditioned medium and ascites fluid.

The morphology of M-cells altered by adding the ascites fluid or the conditioned medium could be reversed to the normal polygonal morphology in about 20 h after the removal of the effectors by changing the culture medium. When the tumor cells were cocultured on M-cell layers in the presence of 25% tumor ascites fluid or when the tumor cells were seeded after the retraction had been induced by adding the ascites fluid, four times as much flattened tumor cell islands were formed as those in the absence of ascites fluid.

Fig. 5. Autoradiograph of a flattened cell island formed 12 h after tumor cell seeding. The tumor cells were prelabeled with L-[4,5-3H]leucine. X 1000.
DISCUSSION

The present preparation of primary cultured layers of mesentery-derived cells consisted mainly of M-cells and was almost free from contamination by macrophages, leukocytes, and fibroblastic cells. Therefore, the cultured mesentery-derived cell layers seemed suitable for studying interactions of AH 130 cells with M-cells. The morphological appearance of M-cells is very similar to those obtained from rat pleura (31) and human ascites fluid (32).

Adhesion of tumor cells to normal cells is no doubt the initial step of invasion by the tumor cells. AH 130 cells, when cultured in plastic culture dishes, did not adhere to substrate and grew in suspension. However, when seeded on M-cell layers, approximately one-half of AH 130 cells adhered to M-cells. Subsequently, some of the adherent cells grew on M-cells to develop piled-up tumor cell nests and some penetrated the M-cell layer to form tumor cell islands. A considerable number of the rest of adherent cells when proliferated appeared to detach from the M-cell layer, since the number of tumor cells in suspension increased to a level more than that which can be predicted from the growth of nonadherent cells (data not shown). The piled-up and invasive tumor cell colonies developed mostly at separate locations on M-cell layer, suggesting heterogeneity of original tumor cell population with respect to invasiveness.

The patterns of tumor cell growth in the culture system (piled-up growth on M-cell layers, invasive growth underneath M-cell layers, and proliferation in suspension) closely resembled their growth in rat peritoneal cavity. In particular, the invasive growth underneath M-cell layers mimics invasion by the tumor cells into rat peritoneum. AH 130 cells isolated from the circulating blood of tumor-bearing animals were found to be highly invasive in vitro. These tumor cells are thought to be the ones that had invaded through mesothelial cell layers into blood vessels. Such remarkable similarity between in vitro and in vivo observation supports the usefulness of our model system in studying the factor(s) that is involved in tumor cell invasion. Haemmerli et al. (33) studied the behavior of V2 carcinoma cells seeded on the surface of an explanted rabbit mesentery, but they did not find any evidence of penetration of the tumor cells into the interior of the mesentery, whereas penetration did occur when the tumor cells were i.p. implanted.

Upon seeding AH 130 cells on the M-cell layer, cellular processes of the tumor cells have been seen protruding under adjacent M-cells (Figs. 4a and 6d). It is presumed that the tumor cells invade by active movement. Similar apparent invasive features have also been reported by others (4, 17, 24, 25, 34). Haemmerli et al. (33) have reported the obvious role in tumor cell invasion of cell locomotion together with other factors like hydrolytic effect and cell proliferation.

The morphological change as evidenced by the partial retraction of M-cells may well be an essential event leading to the penetration of the tumor cells beneath M-cell layers. Although the distinct change was seen at or shortly after the onset of the invasive tumor cell growth, contacts between M-cells could have been broken earlier. Invasion by ascites tumor cells through the peritoneum into various abdominal organs has
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Intercellular junctional structures are often observed between the cells at high cell density (the central zone), whereas fewer junctions are seen at the marginal area. These junctional structures may restrict a large morphological change, but we assume that the intercellular space of M-cells at the central area of culture may also be enlarged after tumor cell seeding, although to a lesser extent.

The retraction of M-cells occurs with the addition not only of the tumor cells but of the tumor ascites fluid or of the medium conditioned by the tumor cell culture, whereas the addition of normal rat serum did not cause any morphological change of M-cells. These events probably indicate that a factor capable of inducing M-cell retraction is produced and excreted by the tumor cells or is generated in culture medium and ascites fluid in the presence of the tumor cells. The effect of the factor does not appear cytotoxic, since the morphological change is reversible. The fact that the formation of the flattened tumor cell islands increased by adding tumor ascites fluid suggests that the factor is involved in tumor cell invasion. We are now attempting to isolate and characterize the factor. Preliminary results indicate that the factor is nondialyzable, heat labile, and perhaps of protein nature. Since the retraction was seen by using plasminogen-depleted culture medium, participation of a plasminogen-activator-plasmin system in the M-cell retraction may well be ruled out. Kimura et al. (15) recently separated from tumor ascites fluid fractions which induced rounding up and exfoliation of mesothelial cells.

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